Rachana Aneja (2010). ***Studies on the catalytic mechanism of plasminogen activation by streptokinase.*** Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
Supervisor: Dr. Girish Sahni Acc. No. : Th-219

SUMMARY OF THE THESIS

Thrombolysis is the 'first line of defense' for the treatment of myocardial infarction and other hemodynamic instability disorders. It involves lysis of the fibrin clot (fibrinolysis) by the serine protease known as plasmin (fibrinolysin) that exists normally in the blood plasma in an inactive form, plasminogen (profibrinolysin).The plasminogen-plasmin reaction is catalyzed by plasminogen activators such as urokinase (UK), tissue plasminogen activator (tPA), streptokinase (SK) and staphylokinase (SAK). The mode of action of bacterial activators, such as SK and SAK is however different from that of physiological activators like UK and tPA, which are proteases and directly cleaves the scissile peptide bond in HPG (Arg561-Val562) and converting it into a bi-chain protease plasmin (HPN). The bacterial activators, however, are not enzymes even though the name ('kinase') suggests so; rather, they bind with a high affinity with either HPG or HPN and modify the substrate specificity of HPN in complex (SK.HPG/HPN) so that the resultant activator enzyme can catalytically transform HPG to HPN.

Although considerable progress has been made in understanding the biology of thrombus formation, pathophysiology of thrombosis and its treatment, all the pharmacologic agents available for prevention or cure offer only modest incremental improvement and are associated with several contraindications. Therefore, it is essential to develop an ideal drug for prophylaxis and treatment of thrombotic disease which targets thrombosis but not hemostasis. The realization of this goal should take into consideration, specific mechanisms and structure-function co-relations associated with different thrombolytic agents. Among the various thrombolytic agents that are being used at present, SK shows the best overall prospects as thrombolytic agents, especially for developing countries since other agents like UK, and tPA, which are physiological HPG activators have lower half-life as compared to SK and are more expensive than SK. The translation of this knowledge from *in vitro* to *in vivo* studies in animal models and further to pharmaceutical development presents opportunities for substantial advances in the prevention and treatment of thrombotic diseases.

The mechanism of action of SK on modulation of substrate-specificity of HPN and activation of the partner molecule HPG/HPN has been investigated over the past few decades. Considerable advancements have been made to identify and characterize

the structural determinants that confer substrate specificity and processivity to SK.HPN complex (Nihalani *et al.*, 1998; Boxrud *et al.*, 2000). SK in complex with partner HPN, expresses new sites other than the active site of the complex called 'exosites', that probably assist in the substrate docking and also might regulate the substrate specificity. Exosite-dependent interactions have been demonstrated for extended interactions between the SK.HPN enzyme complex and the macromolecular substrate HPG (Dhar *et al.*, 2002; Yadav *et al.*, 2008; Tharp *et al.*, 2009). Structural models of the SK.μPN complex, and isolated beta domain of SK (Wang *et al.*, 1998; Wang *et al.*, 1999b) indicate that the SK.HPN enzyme complex interacts with the macromolecular substrate with defined specificity through multiple-site interactions. However, the molecular details of the contributions made by the activator complex SK.HPN, involved in substrate 'recognition' and processivity still needs to be investigated deeper.

In order to identify newer exosites in SK.HPN complex which may be involved in substrate HPG recognition and catalytic turnover, we investigated the importance of the 170-loop especially since the location of the 170 loop in the crystal structure of SK-μPN (Wang *et al.*, 1998; Wang *et al.*, 1999b) suggests that this loop does not likely participate in the formation of the binary complex but is surface-exposed, which hinted at its possible importance in interaction with substrate plasminogen. Therefore, as a first exploratory experiment, a competitive peptide inhibition approach was taken. This was followed by detailed alanine scanning mutagenesis after a positive indication from the peptide inhibition experiments was obtained. The peptide study revealed that disulfide bonded constrained peptide/s corresponding to the primary sequence of the 170 loop distinctly competed with the SK.HPN complex for interaction with substrate HPG. In other words, the synthetic peptides mimicked the solvent-exposed, flexible loop in the beta domain of SK, and thus attenuated its function in HPG activation. Further, the co-factor activity of various SK 170 loop mutants against substrate HPG (human plasminogen) of several alanine mutants was then investigated, employing their 1:1 complexes with human plasmin (HPN). Interestingly, a nearly 10-fold drop in k_{cat} was observed in case of the SK mutant, K180A as compared to wtSK without significant change in the apparent

In order to explore whether all the functionally compromised mutants of the 170 loop are capable of forming active stable 1:1 complexes with partner HPG, their amidolytic activation with HPG at 4°C and 37°C was checked. The results suggested no significant differences in the zymogen activation as compared to wt SK. Similar analyzed. This clearly indicated that the 170 loop of SK beta domain is not involved in the phenomenon of zymogen activation, but is an important substrate interacting exosite. We further checked the cofactor activity of the various 170-loop mutants for the activation of truncated derivative of HPG i.e μPG. The steady-state kinetic parameters for μPG activation revealed that these mutants were compromised in processing of μPG, as indicated by their k_{cat} value, which was 10-fold lower for K180A as compared to wt SK. The substrate affinity of these mutants for substrate μPG remained unchanged. These results are indicative of the fact that the 170 loop interacts specifically with the catalytic domain of substrate HPG as suggested by the proportional decline in k_{cat} of HPG and μPG for various mutants.

Real-time ternary complexation studies using Surface Plasmon Resonance (SPR) were then carried out for ternary binding of either full-length HPG or the isolated serine protease domain of HPG i.e μPG as analytes onto pre-formed SK/SK mutant.HPN complex. The K_d values, obtained for the different 170-loop were not significantly deviant from wtSK.HPN. Overall, these results were in consonance with steady-state kinetic results, in that these mutants are not altered in their ability to recognize and bind substrate HPG but are compromised in processing of substrate. In conclusion, the results revealed the identification of a new exosite, namely the 170 loop in the beta domain of SK, which is involved in the interaction of the activator complex with the serine protease domain of substrate HPG.

SK is a multi-domain protein where the three domains co-operate with each other to drive the macromolecular substrate activation to high levels. The importance of inter-domain co-operativity was earlier studied in our laboratory as well

as by others through the examinationof the enzyme kinetics of individual domains and their different combinations (Sundram *et al.*, 2003; Loy *et al.*, 2001) These studies have suggested that different bi-domain constructs are capable of docking substrate HPG as efficiently as wt SK, but have very poor activities (1-2% as compared to wt SK) even though this is higher compared to individual domains (0.003% of wt SK). One of the possible reasons for the observed inter-domain co-operativity among the three domains is the possibility that there is mechanistic co-operation between the exosites present in each of its three domains, since many studies indicate that the substrate interaction sites are not localized on one or two regions/locales of SK, rather these seem to be scattered over the three domains of SK. These surface exosites might be the underlying reasons for catalytic cross-synchronisation in the three domains, which endows this molecule the highest cofactor activity compared to other bacterial cofactors and also physiological activators. If this is indeed true, and if the identity of all (or nearly all the SK exosites is known), one can reason that a "multi-tiered" mutagenesis approach can be adopted to reveal and validate this phenomenon.

Therefore, we thought of investigating the possible 'global functioning' of surface exosites distributed in the three domains of SK, namely 88-97 loop in the α-domain, 170-loop in the β-domain and the coiled coil region in the γ-domain. In order to assess this, we constructed a series of single-, double-, and triple site mutants comprised of functionally compromised mutants of the three different exosites. Hence, selected representative mutants that were functionally altered were used as building blocks for creating these multi-site mutants. These are, namely ($SK_{88\text{-}97\text{-}BetaTurn}$) in the 88-97 loop (Yadav *et al.*, 2008), K180A of the 170loop (identity revealed during this work), D328G, K3334E and G344D of the coiled coil region of SK (from the studies of Wu *et al.*, 2001; S Yadav, Ph.D. thesis, 2007). The resultant mutants were then examined in detail for their ability to interact with HPG and generate a stable binary complex, leading to functional plasminogen activator capability. The results suggest that the single site mutant namely ($SK_{88\text{-}97\text{-}BetaTurn}$) of the 88-97 loop, which exhibited 3-4 fold decline in HPG activator activity, and K180A mutant of 170 loop showing nearly 10 fold decline, and different mutants of the gamma domain, such as D328G, K334E exhibited varied extents of reduction in HPG activator activity. When double mutants comprised of above mentioned single

HPG activator activity. Triple site mutants carrying all the above mentioned mutants in three exosites simultaneously, such as ($SK_{88\text{-}97\text{-}BetaTurn}$) + K180A+K334E exhibited a nearly 100 fold decline in activity with no significant changes in substrate affinity. This, again, denotes a synergistic effect.

To check whether the multi-site mutagenesis in the three exosites of SK resulted in any changes in the active site micro-chemistry of HPN as compared to SK, their amidolytic parameters for small MW chromogenic substrates were evaluated. The results revealed wtSK-like amidase parameters for all the mutants including the otherwise highly catalytically compromised (for HPG activation) multi-site mutants, suggesting the fact that despite multiple mutations in SK, the binding of mutants with HPN resulted in the preservation of the 'basic' properties of the HPN active site just like the case of wt SK.

The interactions of multi-site mutants of the three exosites with partner HPG and HPN were also examined through active site acylation reaction, as well as their titration with active-site labeled fluorescent HPN – a technique that should reveal any diminution in the pico-molar level binding range at the binary level. The results clearly suggest that these multi-site mutants are fully capable as wt SK in forming active 1:1 complex with HPG as well as retain high affinity with HPN. The real-time binding interactions of SK multisite-mutants. HPN complex(es) with macromolecular substrate, HPG, remained unchanged as evident by K_D value of the mutant enzyme complexes for the macromolecular substrate HPG. Further, steady state kinetics with substrate μPG suggested that these three distinct exosites likely interact specifically with the catalytic domain of substrate HPG. In conclusion, our results suggest that there exists a "distributed network "of exosites in the SK-enzyme complex that, [illegible] through non-covalent interactions in and around the scissile peptide bond area of the catalytic domain of substrate.

The earlier section of the present study, based on solution studies, indicated that the 170-loop of the β-domain of SK constitutes one important "exosite" which contributes substantially to the catalytic turnover of the SK.HPN enzyme complex through its interaction with substrate catalytic domain (Aneja *et al.*, 2009). However, the exact role and identity of participating site/s in the catalytic domain remained unelucidated. In another section, this issue has been addressed through computer-based modeling and docking studies in which substrate miniPG (kringle 5+catalytic domain) was docked to the available crystal structure of SK.μPN enzyme complex. This study revealed that several surface-loops of substrate miniPG are in the proximity of the 170-loop; therefore in the next phase, a multi-site fluorescence resonance energy transfer (FRET) based approach for measuring various prospective enzyme-substrate interaction sites in a 'stabilized' ternary complex was adopted. The FRET studies enabled us to measure the intermolecular distances between one fixed location in the SK 170-loop and five different epitopes/sites spread over the miniaturized substrate derivative, miniplasminogen. The results of the selected ternary interaction model and experimentally determined distances from FRET experiments suggested the most proximal placing of 37-loop near the 170-loop of the β-domain of SK, thus providing the first indication that of the various putative loops in μPG, the 37-loop is closest to the 170-loop of the SK β-domain.

In order to further validate the above results, we pursued a mutational approach wherein different mutants of substrate miniPG were first checked for their ability to get activated by UK. The results suggested that these mutants were fairly activated by UK, which suggested a proper orientation of the scissile peptide area in the mutants of miniPG, as a result of which these could be readily recognized by the direct HPG activator. These miniPG mutants, when analyzed for their zymogen activation capability upon 1:1 complexation with SK suggested that they were amidolytically active and thus capable of forming active enzymatic complexes. This result also tends to suggest that the active-site micro-chemistry of the activated form of these miniPG mutants was not perturbed. Remarkably however, the results of steady state kinetics with 37-loop mutants of catalytic domain, as substrate of the SK.HPN enzyme complex, showed compromised activity. This suggested that the 37

residues Met585, His586, Phe587 in the distal region of the loop. These studies thus clearly succeed in identifying, for the first time, a new locus in the substrate that is important in SK-mediated plasminogen activation.

Thus, the present studies demonstrate in a significant manner, how SK, being a bacterial co-factor of plasmin(ogen), exploits precise multi-point interactions with substrate in order to modulate the catalytic efficiency of its partner HPN/HPG. The specific interaction of SK with substrate HPG is observed to be mediated by several surface exposed loops in both the proteins. It is hoped that the understanding of structure and function of the various exosites in SK.HPN enzyme complex and their cognate interacting loci in substrate will be of practical importance in the design of next-generation plasminogen activators with improved therapeutic potential.